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7.0 BG1Luc ER TA Data Quality

Good Laboratory Practice (GLP) guidelines are nationally and internationally recognized rules designed to produce high-quality laboratory records. GLPs provide a standardized approach to report and archive laboratory data and records, as well as information about the test protocol, to ensure the integrity, reliability, and accountability of a study (EPA 2006b, 2006a; FDA 2009; OECD 1998; Weinberg 2003). This section describes the extent to which the participating laboratories adhered to these guidelines during the validation study, and the effect (if any) of any deviations on the quality of the data. This section also details how often each laboratory failed to generate data that met the plate acceptance criteria (see **Section 4.0**), which necessitated repeat testing during the validation study.

7.1 Compliance with GLP Regulations

The BG1Luc ER TA validation study was conducted according to GLP guidelines at XDS and ECVAM, but not at Hiyoshi, which does not have a formal GLP program. However, prior to initiating the validation study, Hiyoshi provided a guidance document that determined the quality control procedures that they would follow throughout the study. This document is based on the OECD principles of GLP (see **Annex H2)**. In addition, Hiyoshi follows the quality control (QC) and quality assurance (QA) procedures included in the International Organization for Standardization (ISO) 9000 standards, which describe a series of internationally accepted good quality management practices that are applicable to laboratory testing. However, they do not dictate the methods by which those requirements must be met (ISO 2000). ISO 9001-2000, which was used by Hiyoshi, defines and describes requirements for the following standards:

- Quality Management System – requires written quality standards, as well as a control system for all documents and records
- Management Responsibility - assigns the responsibility for all facets of the quality system from creation to improvement, to the organization's senior management and also requires a regular, documented review of the quality program
- Resource Management – requires that personnel must be competent enough to provide quality work and that all facilities, equipment, supporting services, and training programs are sufficient to assure quality product
- Product Realization - requires clear documentation on how design decisions are made, reviewed, validated and controlled

- Measurement, Analysis, and Improvement - requires that all facets of the company be monitored, reviewed, and when necessary, corrected.

7.2 QA Audit Results

GLP compliance in each participating laboratory was determined by an independent QA review of various aspects of the study including:

- Review of protocols and laboratory SOPs
- Review of laboratory operations
- Review of data
- Review of the final report for each testing phase

QA statements that addressed whether the test methods and the results accurately followed the test protocols and that study reports accurately reflected the raw data produced during the study were included in all laboratory reports. The study Project Coordinator and Assistant Project Coordinator also served as secondary QA reviewers for all data and information provided by Study Directors and/or Study Technical Leads. QA review dates for each participating laboratory are provided in **Table 7-1**.

Table 7-1 QA Review Dates

Laboratory	Phase	Review During Testing	Report Review
XDS	1	May-Jul. 2007	Mar. 2008
	2a	Apr. 2008	Nov. 2008
	2b	Sep. 2008	Nov. 2008
	3	Oct. 2009	Jul. 2010
	4	Nov. 2009	Jul. 2010
ECVAM	1	Nov. 2007- Jan. 2008	Mar. 2008
	2a	Oct. 2008	Nov. 2008
	2b	NR	Jan. 2010
	3	NR	Jan. 2010
Hiyoshi	1	Jul. – Oct. 2007	Feb. 2008
	2a	Apr. 2008	Nov. 2008
	2b	Sep. 2010	Feb. 2010
	3	Sep. 2010	Feb. 2010

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; Hiyoshi = Hiyoshi Corporation; NR = not reviewed; XDS = Xenobiotic Detection Systems, Inc.

The QA statements provided in final reports for all validation study phases completed at ECVAM and Hiyoshi (i.e., Phases 1, 2a, 2b, and 3), and Phases 1, 2a, 3, and 4 at XDS indicated that the procedures used to conduct validation study testing followed the test method protocols and that study reports accurately reflected the raw data produced during the study. However, the XDS Phase 2b study report indicated that BG1Luc ER TA antagonist protocol procedures for assessing cell viability were not used in a consistent manner for five (apigenin, atrazine, genistein, *o,p'*-DDT, and resveratrol) of the eight antagonist substances tested. Therefore, testing results from these five Phase 2b substances were not used to evaluate antagonist activity. The validation study Project Coordinator reviewed cell viability assessment procedures with the XDS Study Director and Quality Assurance Officer and apigenin, atrazine, genistein, *o,p'*-DDT, and resveratrol were subsequently retested at XDS. These repeat testing results were then used to evaluate antagonist activity (see **Section 4.0**, **Table 4-12**).

7.3 Test Plate Failure Rates

As described in **Sections 2.7.1.3** and **2.7.2.3**, plate acceptance criteria were established based on results generated in reference standards and control wells. Failures due to results outside of the acceptable range could be an indicator of poor quality data. However, as described in the following sections, some of the plate failures seen may have been due more to overly stringent criteria that were established prior to beginning testing of coded substances in Phase 2a.

7.3.1 Phase 2a

Following Phase 2a of the validation study, the failure rates of plates used during Phase 2a agonist and antagonist testing were evaluated. The percentage of agonist and antagonist test plates that failed acceptance criteria across the participating laboratories were 61% (33/54) and 38% (13/34), respectively:

- At XDS, 53% (8/15) of agonist plates and 43% (6/14) of antagonist plates failed acceptance criteria
- At ECVAM, 80% (24/30) of agonist plates and 50% (7/14) of antagonist plates failed acceptance criteria
- At Hiyoshi, 11% (1/9) of agonist plates and 0% (0/6) antagonist plates failed acceptance criteria

Based on these high failure rates, the plate acceptance criteria were reconsidered to determine if changes to these criteria could reduce the failure rates without compromising the ability of the test method to detect and quantify test substance agonist or antagonist activity. The test plate acceptance criteria that were considered for modification were agonist E2 EC_{50} and methoxychlor RLU control values, and antagonist Ral IC_{50} and flavone control RLU values. Acceptance criteria based on the DMSO control

RLU, agonist E2 reference standard fold induction, and antagonist Ral reference standard fold reduction values were not considered for modification because they are used to monitor background activity (i.e., vehicle control) and reference standard performance (i.e., positive control). The antagonist E2 control acceptance criterion was not considered for modification because it is required for determining test substance antagonist activity.

A comparison was made between qualitative (i.e., positive or negative classification) and quantitative (i.e., EC/IC_{50} values) outcomes for test plates that met all acceptance criteria and those that failed to meet one or more criterion (see **Section 2.7** for Phase 2a acceptance criteria). The results of the qualitative evaluation of the relationship between agonist and antagonist test plate failure rates and acceptance criteria for these parameters are provided in **Tables 7-2** and **7-3** respectively. The qualitative evaluation compared the overall ER TA activity classification of agonist and antagonist test substances for plates that passed and failed acceptance criteria. Results indicate that the ER TA activities (overall positive or negative classification) of substances tested on agonist plates that failed EC_{50} and/or methoxychlor control acceptance criteria and antagonist plates that failed IC_{50} and/or flavone control acceptance criteria were equivalent to the ER TA activities for plates that passed acceptance criteria.

Table 7-2 Phase 2a Test Substance ER TA Agonist Activity for Plates that Passed or Failed Acceptance Criteria[a]

Agonist Test Substance	Laboratory	Passed All Acceptance Criteria	Failed E2 EC_{50} Only	Failed Methoxychlor Only	Failed both E2 EC_{50} and Methoxychlor
Bisphenol A	XDS	POS (3/3)[b]	POS (4/4)	n.a.	n.a.
	ECVAM	POS (3/3)	POS (7/7)	POS (3/3)	n.a.
	Hiyoshi	POS (3/3)	n.a.	POS (1/1)	n.a.
Bisphenol B	XDS	POS (3/3)	POS (4/4)	n.a.	n.a.
	ECVAM	POS (3/3)	POS (4/4)	n.a.	POS (2/2)
	Hiyoshi	POS (3/3)	n.a.	POS (1/1)	n.a.
Corticosterone	XDS	NEG (3/3)	NEG (4/4)	n.a.	n.a.
	ECVAM	POS (3/3)	POS (5/7)	POS (3/3)	n.a.
	Hiyoshi	NEG (4/4)	n.a.	n.a.	n.a.
Diethylstilbestrol	XDS	POS (3/3)	POS (4/4)	n.a.	n.a.
	ECVAM	POS (3/3)	POS (4/4)	n.a.	POS (2/2)
	Hiyoshi	POS (4/4)	n.a.	n.a.	n.a.

Abbreviations: E2 = 17β-estradiol; EC_{50} = half-maximal effective concentration; ECVAM = European Centre for the Validation of Alternative Methods; Hiyoshi = Hiyoshi Corporation; n.a. = not applicable; NEG = negative; POS = positive; XDS = Xenobiotic Detection Systems, Inc.

[a]Agonist activity based on initial classification criteria as defined in **Section 2.7.1.6**

[b]Number in parentheses represents test results (POS or NEG) over the total number of test plates.

Table 7-3 Phase 2a Test Substance ER TA Antagonist Activity for Plates that Passed or Failed Acceptance Criteria

Antagonist Test Substance	Laboratory	Passed All Acceptance Criteria	Failed Ral IC_{50} Only	Failed Flavone Control Only	Failed both Ral IC_{50} and Flavone Control
Dibenzo[*a,h*]anthracene	XDS	POS (3/3)	POS (2/2)	n.a.	n.a.
	ECVAM	POS (3/3)	n.a.	n.a.	n.a.
	Hiyoshi	POS (3/3)	n.a.	n.a.	n.a.
***p*-n-Nonylphenol**	XDS	NEG (3/3)	NEG (3/3)	n.a.	n.a.
	ECVAM	POS (3/3)	n.a.	n.a.	n.a.
	Hiyoshi	POS (3/3)	n.a.	n.a.	n.a.
Progesterone	XDS	POS (3/3)	POS (2/3)	n.a.	n.a.
	ECVAM	POS (3/3)	n.a.	n.a.	n.a.
	Hiyoshi	POS (3/3)	n.a.	n.a.	n.a.
Tamoxifen	XDS	POS (3/3)	POS (3/3)	n.a.	n.a.
	ECVAM	POS (3/3)	n.a.	(1/2)	n.a.
	Hiyoshi	POS (3/3)	n.a.	n.a.	n.a.

Abbreviations: E2 = 17β-estradiol; IC_{50} = half-maximal inhibitory concentration; ECVAM = European Centre for the Validation of Alternative Methods; Hiyoshi = Hiyoshi Corporation; n.a. = not applicable; NEG = negative; POS = positive; XDS = Xenobiotic Detection Systems, Inc.

[a]Agonist activity based on initial classification criteria as defined in **Section 2.7.2.4**

[b]Number in parentheses represents test results (POS or NEG) over the total number of test plates.

The results of the quantitative evaluation of the relationship between agonist and antagonist test plate failure rates and acceptance criteria for agonist E2 EC_{50} and methoxychlor RLU control values, and antagonist Ral IC_{50} and flavone control RLU values are provided in **Tables 7-4.** The quantitative evaluation compared EC_{50} values that could be calculated for bisphenol a, bisphenol b, and diethylstilbestrol at XDS and ECVAM, and the IC_{50} values that could be calculated for tamoxifen at XDS for plates that passed and failed acceptance criteria. Results indicate that agonist substance EC_{50} values from plates that failed EC_{50} and/or methoxychlor control acceptance criteria and tamoxifen IC_{50} values from plates that failed IC_{50} and/or flavone control acceptance criteria were not significantly different from plates that passed acceptance criteria ($p>0.05$).

Table 7-4 Comparison of Phase 2a Test Substance EC/IC_{50} Values for Plates that Passed or Failed Acceptance Criteria

Laboratory and Substance Evaluated	Agonist Plates that Passed All Acceptance Criteria			Agonist Plates that did not Pass E2 EC_{50} and\or Methoxychlor Acceptance Criteria			P Value[1]
	N	Mean EC_{50} Value[2]	SD[2]	N	Mean EC_{50} Value[2]	SD[2]	
XDS/BPA	3	8.8×10^{-2}	7.2×10^{-3}	4	9.9×10^{-2}	1.4×10^{-2}	0.40
ECVAM/BPA	3	1.9×10^{-1}	7.6×10^{-3}	10	1.6×10^{-1}	5.6×10^{-2}	0.16
XDS/BPB	3	3.9×10^{-2}	6.0×10^{-3}	4	4.3×10^{-2}	1.1×10^{-2}	0.63
ECVAM/BPB	3	4.2×10^{-2}	1.3×10^{-2}	4	7.5×10^{-2}	1.7×10^{-2}	0.06
XDS/DES	4	1.4×10^{-5}	5.0×10^{-6}	4	2.6×10^{-5}	1.1×10^{-5}	0.20
Laboratory and Substance Evaluated	**Antagonist Plates that Passed All Acceptance Criteria**			**Antagonist Plates that did not Pass Ral\E2 IC_{50} and\or Flavone Acceptance Criteria**			**P Value[1]**
	N	**Mean IC_{50} Value[2]**	**SD[2]**	**N**	**Mean IC_{50} Value[2]**	**SD[2]**	
XDS/TAM	4	1.5×10^{-1}	5.7×10^{-2}	3	3.1×10^{-1}	8.8×10^{-2}	0.11

Abbreviations: BPA = bisphenol A; BPB = bisphenol B; DES = diethylstilbestrol; E2 = 17ß-estradiol; EC_{50} = half–maximal effective concentration; IC_{50} = half maximal inhibitory concentration; ECVAM = European Centre for the Validation of Alternative Methods; Hiyoshi = Hiyoshi Corporation; Methoxychlor = weak positive methoxychlor control; N = number of plates; Ral = raloxifene HCl; SD = standard deviation; TAM = tamoxifen; XDS = Xenobiotic Detection Systems, Inc.

[1]P>0.05 indicates that EC_{50} or IC_{50} values are not significantly different

[2]All are expressed in EC_{50} values (µg/mL) except for XDS/TAM, which is expressed in IC_{50} values (µg/mL)

Based on this evaluation, it was determined that test plate acceptance criteria based on agonist E2 EC_{50} and methoxychlor RLU control values, and antagonist Ral IC_{50} and flavone control RLU values could be eliminated without compromising the ability of the test method to detect and quantify test substance agonist or antagonist activity. The modified acceptance criteria for agonist and antagonist comprehensive testing are provided in **Sections 2.7.1.3** and **2.7.2.3** respectively and were used for all plates tested in the remainder of the validations study (i.e., Phases 2b, 3, and 4).

7.3.2 Phases 2b, 3, and 4 Failure Rates

The plate failure rates for the remaining phases of the study are provided in **Tables 7-5** and **7-6**. Results indicate that the modified acceptance criteria based on Phase 2a results significantly reduced the failure rates of agonist test plates in Phases 2b, 3 and 4 (≤ 27%) compared to the Phase 2a agonist test plate failure rate (61%). The failure rate of Phase 2b antagonist test plates (14%) was also significantly reduced

compared to the Phase 2a antagonist test plate failure rate (38%); failure rates during Phase 3 and 4 antagonist were only marginally decreased (36% and 35%, respectively).

Table 7-5 Test Plate Failure Rates for Agonists; Phases 2b-4

Phase	Laboratory	% of Plates that Failed Acceptance Criteria[a]
2b	XDS	0% (0/13)
	ECVAM	25% (4/16)
	Hiyoshi	19% (3/16)
	Total	16% (7/45)
3	XDS	26% (12/47)
	ECVAM	29% (10/35)
	Hiyoshi	0% (0/34)
	Total	19 % (22/116)
4	XDS	27% (11/41)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; Hiyoshi = Hiyoshi Corporation; XDS = Xenobiotic Detection Systems, Inc.

[a]Number in parentheses represents the number of test plates that failed acceptance criteria over the total number of plates tested.

Table 7-6 Test Plate Failure Rates for Antagonists; Phases 2b-4

Phase	Laboratory	% of Plates that Failed Acceptance Criteria
2b	XDS	0% (0/12)
	ECVAM	33% (6/18)
	Hiyoshi	0% (0/14)
	Total	14% (6/44)
3	XDS	47% (28/59)
	ECVAM	31% (11/36)
	Hiyoshi	13% (3/24)
	Total	36% (43/119)
4	XDS	35% (8/23)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; Hiyoshi = Hiyoshi Corporation; XDS = Xenobiotic Detection Systems, Inc.

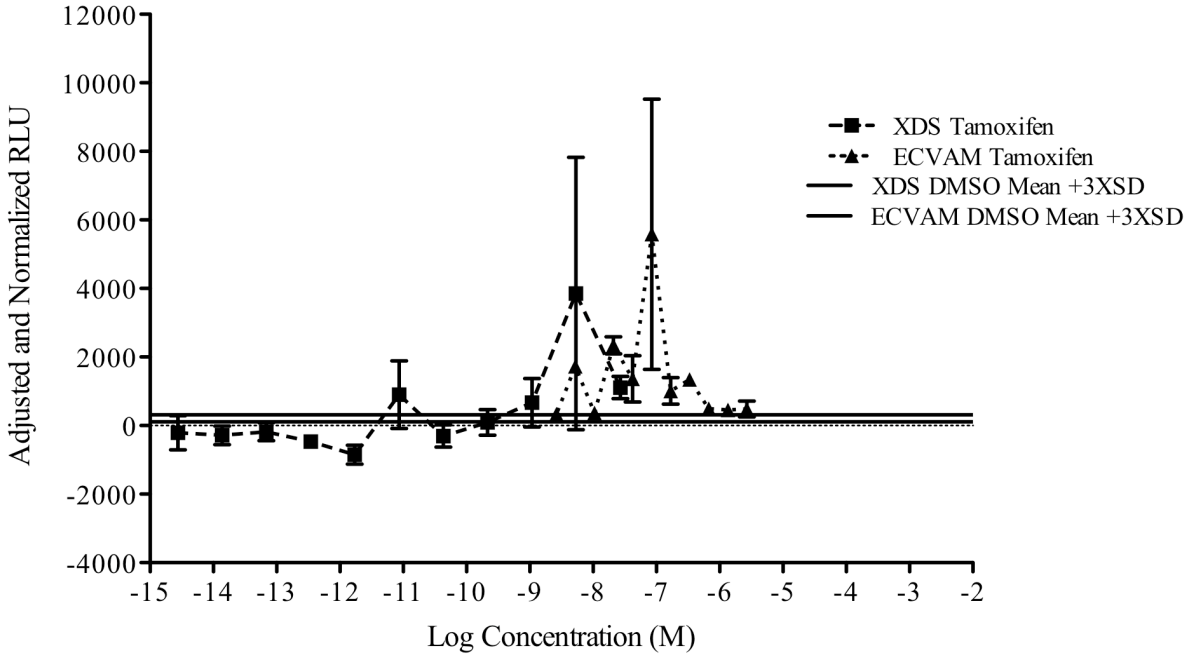
[a]Number in parentheses represents the number of test plates that failed acceptance criteria over the total number of plates tested.

7.4 Inadequate Results

As described in **Section 2.0**, test substances were classified as positive, negative, or inadequate based on updated test method decision criteria. Inadequate data were identified as such based on those substances that failed to meet the decision criteria for either a positive or negative response defined in **Section 2.12.3**. The classification of data as "inadequate" is due to poor quality data that could not be interpreted as valid because of major qualitative or quantitative limitations. Normally, substances with "inadequate" data would be retested and conclusive results would therefore be expected for all test substances. However, since the updated classification system was developed after testing was complete, these substances were not retested.

As an example, tamoxifen test results at XDS and ECVAM failed to produce a clear concentration response curve and the resulting data had overlapping error bars due to one or more highly variable results (**Figure 7-1**).

Figure 7-1 Inadequate Test Results: Tamoxifen Tested at XDS and ECVAM:



[a]Each point represents the mean adjusted and normalized RLU value and SD from triplicate wells.

While the actual test substance classifications based on BG1Luc ER TA results are presented in **Tables 4-11** and **4-12** (see **Section 4.0**), the frequency of inadequate data produced at each laboratory is summarized in **Table 7-7**. Inadequate test results in the agonist test method occurred from 3% (1/40) at

Hiyoshi up to 27% (11/41) at XDS. Antagonist testing produced far fewer inadequate results (3% to 5% of tests) but again, Hiyoshi produced the fewest inadequate results.

Table 7-7 Summary of Test Results Classified as Inadequate

Phase	Laboratory	Agonist[a]	Antagonist
Phase 2	XDS	0% (0/12)	0% (0/12)
	ECVAM	0% (0/12)	0% (0/12)
	Hiyoshi	0% (0/12)	0% (0/12)
Phase 3	XDS	27% (11/41)	5% (2/41)
	ECVAM	17% (7/41)	5% (2/41)
	Hiyoshi	3% (1/40)	3% (1/41)
Phase 4	XDS	16% (4/25)	4% (1/25)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; Hiyoshi = Hiyoshi Corporation; XDS = Xenobiotic Detection Systems, Inc.

[a]Number in parentheses represents the number of inadequate results over the total number of substances tested.

7.5 Availability of Laboratory Notebooks or Other Records

All records are stored and archived by the participating laboratories and are available for inspection. The raw data for each test (in EXCEL® and PRISM® files) are available upon request from NICEATM on compact disc(s). Requests can be made by mail, fax, or e-mail to Dr. William S. Stokes, NICEATM, NIEHS, P.O. Box 12233, MD EC-17, Research Triangle Park, NC, 27709, (phone) 919-541-2384, (fax) 919-541-0947, (e-mail) niceatm@niehs.nih.gov.

EPA. 2006a. Good Laboratory Practice Standards. Federal Insecticide, Fungicide and Rodenticide Act. 40 CFR 160.

EPA. 2006b. Good Laboratory Practice Standards. Toxic Substances Control Act. 40 CFR 792.

FDA. 2009. Good laboratory practice for nonclinical laboratory studies. 21 CFR 58.

ISO. 2000. Quality Management Systems - Requirements. Geneva:International Organization for Standardization.

OECD. 1998. OECD Series on Good Laboratory Practice and Compliance Monitoring No. 1 - OECD Principles on Good Laboratory Practice (as revised in 1997). Paris:OECD Publishing. Available: http://www.oecd.org/document/63/0,3343,en_2649_34381_2346175_1_1_1_1,00.html

Weinberg S. 2003. Good Laboratory Practice Regulations. Third Edition, Revised and Expanded ed. New York, U.S.A.:Marcel Dekker, Inc.